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Title: Increased CD40 ligation and reduced BCR signalling leads to higher IL-10 production in B-cells from tolerant kidney transplant patients

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Authorship

Author contribution: EN-L processed the samples used in this study, designed the experiments, performed the experiments, analysed the data and prepared the manuscript. PC and RM carried out Luminex analysis. PM, MR and YK processed GAMBIT samples. EN-L, GL, RIL and MH-F wrote the manuscript. GML, RIL, GL and MH-F obtained funding. MH-F directed the project. GAMBIT Consortium collaborators provided patient samples and detailed clinical information.

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Abbreviation

BCR: B-cell receptor

CD: Cluster of differentiation

CD40L: CD40 ligand

ERK1/2: Extracellular Signal-Regulated Kinases 1 and 2

MEK: Mitogen-Activated Protein Kinase Kinase

PMA: Phorbol-12-Myristate-13-Acetate

TCR: T-cell receptor

Abstract

Background: An increased percentage of peripheral transitional B-cells producing IL-10 has been observed in patients tolerant to kidney allografts. In healthy volunteers, the balance between the CD40 and B-cell receptor (BCR) signalling modulated IL-10 production by B-cells, with stimulation via the BCR decreasing CD40-mediated-IL-10 production. In this study, we evaluate whether in tolerant kidney transplant patients the increased IL-10 production by B-cells was due to an altered CD40 and/or BCR signalling.

Methods: B-cells obtained from a new cohort of tolerant renal transplant recipients and those from age- and gender-matched healthy volunteers, were activated via CD40 and BCR, either alone or in combination.

Results: In tolerant patients we observed higher percentages of B-cells producing IL-10 after CD40 ligation and higher expression of CD40L on activated T-cells, compared to healthy controls. Furthermore, B-cells from tolerant recipients had reduced ERK signalling following BCR-mediated activation compared to healthy controls. In keeping with this, combining BCR signalling with CD40 ligation did not reduce IL-10 secretion as was observed in healthy control transitional B-cells.

Conclusion: Altogether our data suggests that the altered response of B-cells in tolerant recipients may contribute to long-term stable graft acceptance.

Introduction

A state of operational tolerance has been described in kidney transplant patients who deliberately decided to stop immunosuppression¹. These patients displayed a stable graft function after complete drug withdrawal for longer than one year. In 2007, Brouard *et al.* performed the first study in kidney transplant patients aimed at identifying biomarkers of tolerance from peripheral blood samples, defining a “tolerant fingerprint” of 49 genes². The same group reported later that tolerant recipients exhibited a significant increase in absolute numbers and frequency of total B-cells, particularly activated memory and early memory B-cells³. In the same year, two independent multicentre studies, the European IOT study⁴ and the USA ITN study⁵ reported and validated a B-cell signature in 11 and 19 kidney transplant tolerant patients, respectively. In both studies an over-expression of multiple B-cell related genes and maintenance of peripheral blood B-cell numbers and percentages in tolerant recipients were observed. Moreover, in the ITN study an up-regulation of *CD20* mRNA in sediment cells from urine, and elevated numbers of peripheral blood naïve and transitional B-cells in tolerant participants, compared with those receiving immunosuppression, were also observed⁵. In 2012, another group reported that tolerant recipients exhibited similar numbers and percentages of circulating total B-cells, naive, memory and regulatory B-cells than healthy individuals, as well as preserved B-cell receptor repertoire. In addition, they demonstrated that tolerant recipients displayed a conserved capacity to activate CD40/STAT3 signalling pathway in regulatory B-cells⁶. In 2014, Brouard’s group showed that B-cells from tolerant recipients exhibited a defective expression of factors involved in the differentiation into plasma cells and the B-cells were more prone to cell death by apoptosis compared to patients with stable graft function⁷. Finally in 2015, the same

group showed that tolerant recipient exhibit a higher number of Granzyme B⁺ B-cells compared to healthy volunteers and stable recipients⁸. They showed that Granzyme B⁺ B-cells were able to inhibit CD25⁺CD4⁺Tcell responses through a pro-apoptotic mechanism⁸.

CD40 and BCR ligation on B-cells are key events in their function and T-cells contribute to both. The predominance of the BCR signalling alone favours apoptosis⁹, whereas the predominance in the CD40 ligation favours cell survival⁹ and IL-10 secretion¹⁰. The combination of both signals favours cell activation, differentiation and antibody production¹¹, however it has been reported that IL-10 production by CD27⁺B-cells is reduced when CD40 and BCR are triggered together compared to CD27⁺B-cells activated only through CD40-CD40L interaction^{10,11}.

In this study we hypothesised that altered BCR/CD40 signalling is linked to increased IL-10 production observed in tolerant patients. The B-cell responses from a cohort of tolerant renal transplant recipients were compared with age/gender-matched healthy volunteers. The data presented in our study demonstrated that B-cells from tolerant patients exhibited an imbalance in CD40/BCR signalling compared to healthy controls, suggesting that these differences may contribute their increased IL-10 production and to the long-term graft survival observed in tolerant patients.

Materials and Methods

Patients

Patient samples were donated from the Genetic Analysis & Monitoring of Biomarker of Immunological Tolerance (GAMBIT) study, approved by the Institute of Child Health/Great Ormond Street Hospital Research Ethics Committee 09/H0713/12. All experiments were performed in accordance with the approved guidelines and regulations. Samples were processed and analysed in a blinded fashion after informed consent was obtained from all subjects. Of patients from the GAMBIT study, the following ones have been used in this project:

Tolerant (n=16): Functionally stable kidney transplant recipients despite having stopped all their immunosuppression for longer than one year with a serum creatinine CRT < 160umol/l and < 10% rise in the last 12 months. ESRF causes have been summarised in SupplTable 1. **Healthy control (n=11):** Healthy volunteers were age and gender-matched to tolerant patients.

Patient characteristics are described in Table 1.

Sample Collection

PBMCs from patients and healthy controls were isolated from peripheral blood by Ficoll-Hypaque (PAA) density gradient centrifugation. Cells were re-suspended in 10% DMSO (Sigma-Aldrich)-AB human serum (BioWest) and frozen immediately at -80°C. After 24hrs, cells were transferred into liquid nitrogen where they were kept until use. Cell counts for each patient were obtained from their respective full blood count test (FBC; NHS). Serum samples were obtained after centrifugation of coagulated peripheral blood samples (2000RPM/20min/room temperature), stored at -80°C and kept until use.

B-cell phenotypic studies

PBMC samples were thawed from liquid nitrogen on the same day as the staining and 3×10^6 PBMCs were used to study B-cell phenotype. PBMCs were stained with Live/Dead (Invitrogen), anti-IgM-PerCP-Cy5.5 (BD), anti-IgD-PE (BD), anti-CD19-AlexaFluor780, anti-CD20-AlexaFluor700, anti-CD27-APC, anti-CD24-FITC, anti-CD38-PECy7 (all eBioscience) for 30min at 4°C. Cells were acquired on LSRFortessa (BD). Data was acquired until at least 5,000 events in the B-cell subset were counted. All flow cytometry data was analysed using FlowJo (Tree Star, Inc. Ashland, OR 97520 USA) or DIVA software (BD).

B-cell functional assays in response to CD40L activation measured in kidney transplant patient and healthy control's PBMCs

1×10^6 PBMCs from patient samples were cultured with or without 0.5×10^5 plate bound CD40L-transfected and non-transfected mouse L-fibroblast cells (X-ray irradiated for 30min 9,045 cGy) for 72hrs. After 3 days, expression of CD86-FITC and CD25-PE (all eBioscience) and intracellular IL-10-PE (BD) and TNF- α (eBioscience) were measured in CD20⁺B-cells. Supernatants were collected and stored at -80°C to subsequently measure cytokines by ELISA. Methods for intracellular staining, ELISA and experiments in B-cell subsets isolated from healthy leukocytes retained in filtering cones and patient samples can be found in the supplementary content.

T-cell activation

CD4⁺T-cells were isolated using CD4⁺T-cell Isolation Kit II, human (Miltenyi Biotec). Cells were activated with α CD3/CD28 beads (1:2 ratio) (Invitrogen) for 8 and 48hrs. After 8hrs, cells were stained with CD4-FITC (eBioscience) and CD40L-PE (eBioscience) for 30min at 4°C. Cytokines in the supernatants were measured using Cytometric Beads Array human Th1/Th2/Th17 kit (BD) For IL-2: the sensitivity of the

kit was 2.6 pg/mL and the standard curve range was from 2.6 to 5000 pg/mL. For IL-4: the sensitivity of the kit was 4.9 pg/mL and the standard curve range was from 4.9 to 5000 pg/mL. Cells and beads were acquired on LSRFortessa (BD).

BCR activation measured on patient's PBMCs

PBMCs from patients and healthy controls were thawed and rested overnight in media supplemented with IL-2 (100ng/ml, R&D) at 37°C 5% CO₂. The next day, cells were stained with CD20-Pacific Blue (eBioscience), CD27-APC (eBioscience), CD24-PE (eBioscience), CD38-PE-Cy7 (eBioscience) and Live/Dead dye (Invitrogen) for 20min at 37°C 5% CO₂. After the staining, 1x10⁶ PBMCs were placed in FACS tubes, cells were washed, rested for 30min at 37°C 5% CO₂ and activated with anti-IgM (20µg/ml)/anti-IgG (20µg/ml) (Southern Biotech) for 0, 10 and 30min and with PMA (0.1µM, SIGMA) for 10min at 37°C. Cells were fixed with BD Cytofix buffer (BD) for 10min at 37°C, permeabilized with BD Phosflow Perm Buffer III (BD) for 30min on ice and stained with anti-ERK1/2 (pT202/pY204)-AlexaFluor488 (BD) for 30min at 4°C. Cells were acquired on LSRFortessa (BD).

Detection of IgG1 serum levels

Serum IgG1 levels were measured with the Human Immunoglobulin Isotyping Magnetic Bead Panel Milliplex Map Kit (Millipore) in healthy volunteers and tolerant recipients.

Statistical analysis

All columns in graphs represent the mean and standard error of mean. Non-parametric Mann-Whitney test was used to analyse phenotypic flow cytometry data in patient and healthy control samples. Two-way Repeated Measurement (RM) ANOVA test with Sidak's multiple comparisons was used to evaluate differences between non-activated and activated samples. We defined significance as ****

$P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$. We performed data tables, statistical analysis results and graphs using Prism 6 (1992-2012 GraphPad Software, Inc La Jolla, CA 92037 USA).

Results

Tolerant kidney transplant recipients exhibit higher percentages of IL10⁺ B-cells after CD40L activation compared to healthy volunteers

Previous studies, from us and others, have established that the percentages of total B-cells in tolerant renal transplant patients were similar to healthy volunteers^{3,5,6}.

This was confirmed in a new cohort of tolerant recipients (Table.1) in whom similar percentages of total, memory, naïve and transitional B-cells between healthy controls and tolerant recipients were observed (Fig.1A and Fig.S1).

IL-10 production has been shown to be the main regulatory mechanism for transitional B-cells in autoimmunity¹², GVHD¹³, as well as transplantation^{5,7,14}, therefore, the production of this cytokine was studied in the new cohort of tolerant patients. B-cells were stimulated via CD40 molecules by using CD40L-expressing L-cells, and as control non-transfected L-cells. CD40 stimulation of B-cells, in healthy volunteers sorted B-cell subsets isolated from peripheral blood cones, preferentially induced IL-10 production by transitional B-cells, while the TLR9 agonist CpG, used as positive control, led to IL-10 production by all the different B-cell subsets (Fig.S2). After confirming that CD40-ligation was inducing IL-10 mainly in transitional B-cells, the production of IL-10 was then compared in B-cells between healthy volunteers and tolerant patients. The percentage of IL-10⁺B-cells and the levels of IL-10 in the supernatants were significantly higher in samples from tolerant recipients compared to healthy volunteers, while no differences in the levels of TNF- α between the two groups was observed (Fig.1B). To confirm that the difference in IL-10 production between tolerant patients and healthy individuals was not due to differences in the susceptibility of B-cells to be activated, the expression of CD25 and CD86 molecules by B-cells after CD40-ligation was analysed. Similar up-regulation of both molecules

was observed in the two cohorts of individuals (Fig.1C).

T-cells are key in the activation of B-cells and the engagement of CD40 expressed by B-cells by CD40L on T-cells is essential in inducing IL-10 production¹². Therefore, we measured the expression of CD40L on CD4⁺T-cells from tolerant patients and healthy controls. We observed that the levels of expression of CD40L, while similar between the two cohorts of individuals before T-cell activation, were higher 8hrs-post activation with CD3/CD28 beads, in T-cells from tolerant patients, suggesting that T-cells from tolerant recipients have an advantage in engaging CD40 molecules on B-cells compared to healthy volunteers (Fig.1D).

B-cells from tolerant recipients have decreased BCR signalling compared to healthy volunteers.

We have shown so far that tolerant patients exhibited increased IL-10 production by B-cells and that CD40L expression is higher in T-cells compared to healthy volunteers. Another function of B-cells that may be relevant for the maintenance of a tolerant state, is their capacity to bind antigen via the BCR and then present it to T-cells¹⁵. At the same time, it has been shown previously that BCR engagement leads to the maturation and activation of B-cells via the MEK-ERK signalling pathway¹⁶. Therefore, the phosphorylation of ERK was evaluated by stimulating B-cells with anti-IgG/anti-IgM (Fig.2A and B). Activation with PMA, that bypasses the BCR, was used as a positive control (Fig.2B). The results are presented as the differences in phosphorylation between samples activated via the BCR for 10min and non-activated (Δ ERK) (Fig.2C). All B-cell subsets from tolerant recipients exhibited reduced Δ ERK compared to healthy controls (Fig.2D), while no differences in Δ ERK were observed after PMA activation (Fig.2E), suggesting that the reduction in ERK

phosphorylation in the B-cells from tolerant recipients was specific to the BCR stimulation. CD40-mediated IL-10 production by CD27⁺B-cells was inhibited with simultaneous CD40 and BCR stimulation¹⁰. As predicted, IL-10 production was reduced in transitional B-cells from healthy individuals when B-cells were stimulated via CD40 and BCR compared to ligation of CD40 alone (Fig.2F and Fig.S3). In addition, we observed that the combination of CD40 and BCR stimulation resulted in down-regulation of ERK-phosphorylation compared to BCR stimulation alone (Fig.S4). Having shown that the effect on IL-10 release was mainly observed in the transitional population, IL-10 production by transitional B-cells in response to ligation of CD40 and BCR was compared between healthy individuals and tolerant patients. We found that, whereas IL-10 production following CD40 and BCR stimulation was decreased in transitional B-cells from healthy volunteers, it was maintained in transitional B-cells from tolerant recipients (Fig.2G). These results suggest that the reduced BCR signalling present in B-cells from tolerant recipients allowed sustained CD40-mediated IL-10 production after dual activation.

It has been described that mice deficient for Ras guanyl nucleotide exchange factor 3 (*Rasgrp3*^{-/-}), exhibit reduced ERK phosphorylation after BCR activation and lower titers of IgG1 in their sera¹⁷, suggesting that reduction in the BCR-ERK signalling plays a role in the humoral response, skewing it to IgG subclasses with lower complement binding. Furthermore, IL-2 and IL-4 present during the class-switch also affect the production of IgG1^{18,19}. Therefore, the production of these two cytokines by CD4⁺T-cells from patients and healthy volunteers was measured after TCR activation. The analysis of these cytokines after 48hrs of culture revealed that the levels of IL-2 and IL-4 were lower in T-cells from tolerant patients compared to healthy controls (Fig.2H). In accordance with reduced BCR signalling and reduced

levels of IL-2 and IL-4, lower levels of IgG1 were found in the serum of tolerant recipients compared to healthy volunteers (Fig.2I).

Discussion

Here we describe for the first time that tolerant patients exhibit higher percentages of IL-10-producing B-cells after CD40 activation and reduced BCR signalling compared to healthy controls. We also demonstrated that the reduction in BCR signalling favoured IL-10 production in the transitional B-cell population of tolerant recipients. Furthermore, the reduced ERK activation together with the reduced IL-2 and IL-4 production by T-cells may contribute to the low IgG1 levels observed in the serum (Fig.S5).

The percentages of B-cells and the expression of B-cell related genes has been previously compared between tolerant patients and either healthy individuals or other groups of patients under immunosuppressive therapy³⁻⁶. Newell *et al.* reported for the first time that following stimulation with PMA+Ionomycin, transitional B-cells from tolerant individuals expressed higher percentages of IL-10 relative to stable patients or healthy controls⁵. Although their differences were significant, they recognised that the overall percentages of IL-10 producing B-cells were extremely low, with many samples containing no IL-10-producing cells, and with a large overlap in IL-10 expression between groups, suggesting that PMA+Ionomycin may not have been the optimal stimulus to measure IL-10-producing B-cells within PBMCs. On the other hand, data from Silva *et al.* using specific CD40 activation, showed higher STAT-3 phosphorylation in regulatory B-cells from 5 tolerant patients compared to patients with chronic rejection, indicating that the IL-10 signalling pathway was activated in tolerant recipients⁶. More recently, Chesneau *et al.*, demonstrated higher IL-10 production by B-cells from tolerant kidney transplant patients compared to healthy volunteers and stable recipient after a combination of anti-IgG/IgM/IgA, soluble CD40L, CpG and IL-2⁷. However, the addition of CpG in the latter study complicated

the interpretation of the results, as CpG induced IL-10 production by a different signalling pathway compared to CD40 and by different B-cell subsets (Fig.S2). Here we showed that CD40L activation induces IL-10 production preferentially by transitional B-cells and that IL-10 production was higher in transitional B-cells from tolerant patients after dual-BCR/CD40L activation compared to healthy volunteers. Since these activations targeted mainly the transitional B-cell population, it is difficult to compare the IL-10 production between tolerant recipients and patients under immunosuppressive treatment as the latter patients exhibited extremely low percentages of transitional B-cells (data not shown).

IL-10 is a well-known anti-inflammatory cytokine. The production of IL-10 by B-cells has been shown to inhibit T-cell proliferation¹³, cytokine production¹² and promote regulatory T-cell differentiation²⁰. Furthermore, IL-10 also attenuates the antigen presentation capacity of B-cells by down-regulating the expression of CD86 on their surface in mouse²¹ and human¹⁴. In human diseases, IL-10 production has been proposed as the main regulatory mechanism used by B-cells in lupus¹², arthritis²⁰, graft-versus-host disease (GVHD)¹³ and transplantation²². The production of IL-10 in tolerant recipients may indicate that in these patients it may contribute to the maintenance of a stable graft function in the absence of any immunosuppressive therapy.

Along with more IL-10 production, B-cells from tolerant recipients also exhibited a reduced BCR signalling. Mice with impaired BCR signalling exhibited lower titers of IgG1 in the sera compared to wild-type mice¹⁷ suggesting that reduced BCR signalling affects the Ig class-switch to IgG1. Furthermore, Chesneau *et al.* showed that B-cells from tolerant recipients exhibit a down-regulation of B-cell differentiation genes⁷. Accordingly, we observed in tolerant patients lower levels of IgG1 in the sera

and lower secretion of the cytokines that contribute to the IgG class switching, IL-2 and IL-4 compared to healthy volunteers. More specifically, IL-4 has been shown to induce the production of the IgG subclasses IgG1, IgG2 and IgG3, while IL-2 promotes IgG1 and IgG3 release^{18,19}. In addition, IL-2 produced by T-cells is required for ERK1/2-mediated differentiation of human naive B-cells into plasma cells²³ and to enhance the effect of IL-10 for the induction of antibody production²⁴. Furthermore, it has been shown that low levels of CD40 stimulation induced B-cell activation, whereas high levels of CD40 ligation inhibited Ig production¹¹. Our results suggest that the imbalance in the BCR/CD40 signalling pathway and reduced IL-2 and IL-4 secretion by T-cells may have prevented the class-switch and further secretion of IgG1 in these patients. In the context of transplantation, reduced levels of IgG1 decreased complement dependent cytotoxicity and Fc-receptors binding, therefore impairment in the humoral response of these patients could have promoted the tolerant state by reducing antibody-dependent cellular cytotoxicity²⁵. Altogether our data suggest that the type of responses produced by B and T-cells in tolerant recipients may contribute to long-term stable graft function. Further studies are required to understand whether these responses were developed after transplant, as allograft-specific responses, or whether they are intrinsic or genetically determined in these patients.

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Table 1

Patient Data	Healthy Control	Tolerant
Number of patients	11	16
Age in years [mean (range)]	48.0 (23-72)	50.6 (22-77)
Gender (Male/Female)	(9/2)	(13/3)
Recipient age at Transpl. [mean (range)]	-	32.1 (11-56)
Donor (Living/Deceased)	-	(9/7)
Time post Transpl. in years [mean (range)]	-	18.4 (10-34)
Years immunosuppression free [mean (range)]	-	6.0 (1-23)
HLA mismatches		
<i>Number of patients with:</i>		
-No mismatches	-	6
-HLA (A or B) mismatches	-	2
-HLA (A + B) mismatches	-	1
-HLA (A + DR) mismatches	-	0
-HLA (B + DR) mismatches	-	0
-HLA (A + B + DR) mismatches	-	6
-Missing data	-	1
Donor-specific antibodies (DSA)		
<i>Number of patients with:</i>		
-No DSA	-	14
-DSA Class I	-	1
-DSA Class II	-	1
-DSA Class I and II	-	0
Renal Function Parameters		
Creatinine (mmols/L) [mean]	-	117.2 \pm 15.6
eGFR (mL/min/1.73m ²) [mean]	-	60.9 \pm 16.5
Cell Count		
Lymphocytes count x 10 ⁹ [mean \pm SD]	2.20 \pm 0.55	2.30 \pm 0.71
B-cell count x10 ⁹ [mean \pm SD]	0.43 \pm 0.24	0.46 \pm 0.21
Memory B-cell count x10 ⁹ [mean \pm SD]	0.12 \pm 0.10	0.11 \pm 0.07
Naïve B-cell count x10 ⁹ [mean \pm SD]	0.34 \pm 0.23	0.38 \pm 0.16
Transitional B-cell count x10 ⁹ [mean \pm SD]	0.03 \pm 0.03	0.05 \pm 0.02

Table 1: Clinical data of tolerant kidney transplant recipients and healthy volunteers.

DSA: donor-specific antibodies. eGFR: *estimated glomerular filtration rate*.

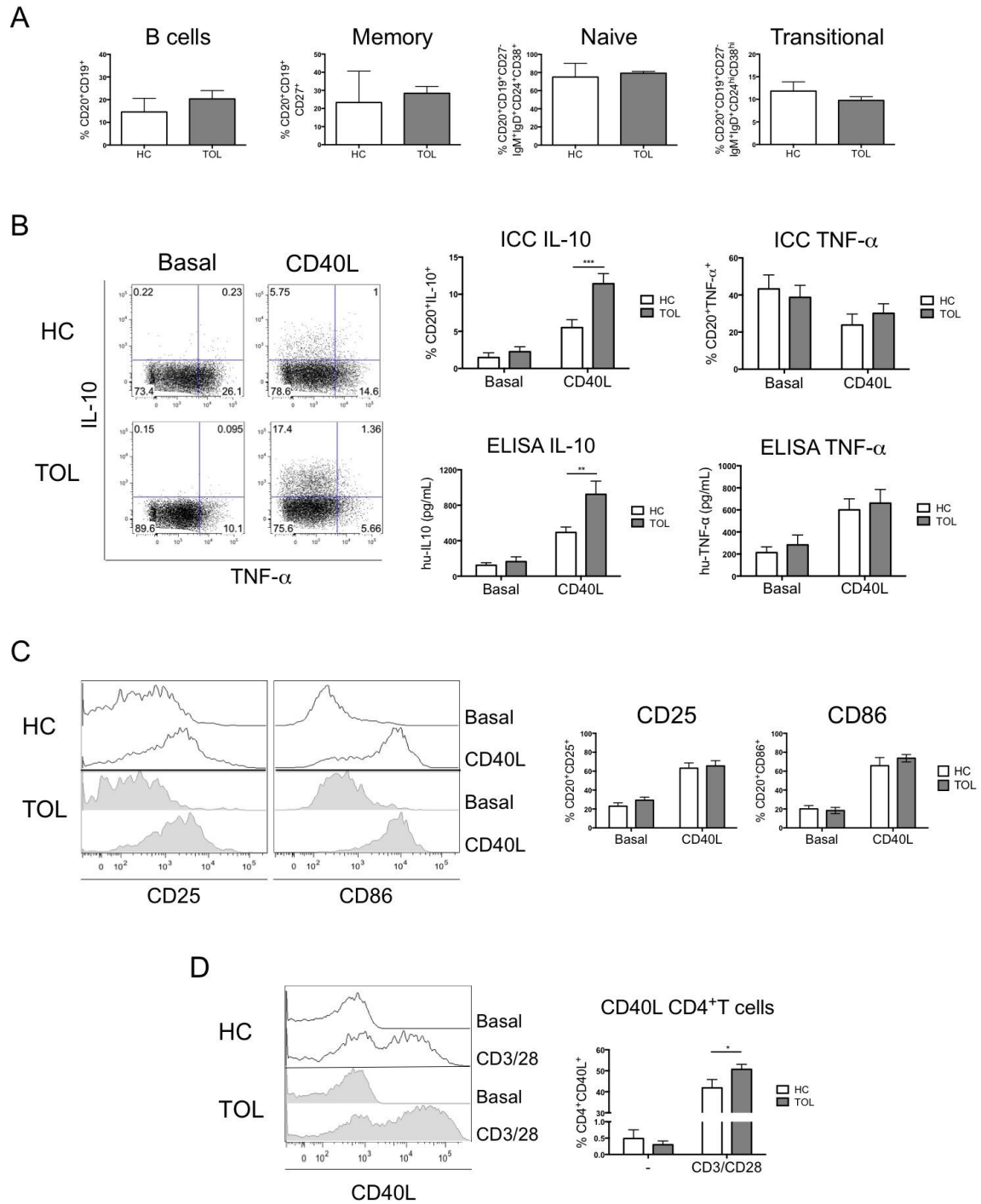


Fig. 1: Higher IL-10 production by B-cells from tolerant recipients was observed after CD40 ligation

A) Comparison of the distribution of total, memory, naïve and transitional B-cells within PBMCs between healthy control (HC=11) and tolerant (Tol=16) patients. Total

B-cells were identified as CD20⁺CD19⁺, memory B-cells as CD27⁺, naïve B-cells as CD27⁻IgD⁺IgM⁺CD24⁺CD38⁺ and transitional B-cells as CD27⁻IgD⁺IgM⁺CD24^{hi}CD38^{hi} by surface staining. A Mann-Whitney test was used. B) Production of IL-10 and TNF- α by intracellular staining and protein levels were measured after 3 days of activation with 0.5x10⁵ non-transfected L-cells (Basal) or human-CD40L-transfected L-cells (CD40L) in 1.0x10⁶ PBMCs from HC=8 and Tol=10 patients. Detection limit for IL-10 and TNF- α were 2 and 15.6pg/ml, respectively. C) The expression of CD25 and CD86 on CD20⁺ B-cells was measured after 3 days of activation with 0.5x10⁵ non-transfected L-cells (Basal) or human-CD40L-transfected L-cells (CD40L) in 1.0x10⁶ PBMCs from HC=11 and Tol=14 patients. D) Up-regulation of CD40L in CD4⁺T-cells from healthy HC=6 and Tol=7 patients were measured after 8hrs of activation with CD3/CD28 beads (1:2 ratio). Two-way RM ANOVA test with a Sidak's multiple comparisons test was used. For all statistical tests **** $P<0.0001$, *** $P<0.001$, ** $P<0.01$ and * $P<0.05$ were considered significant.

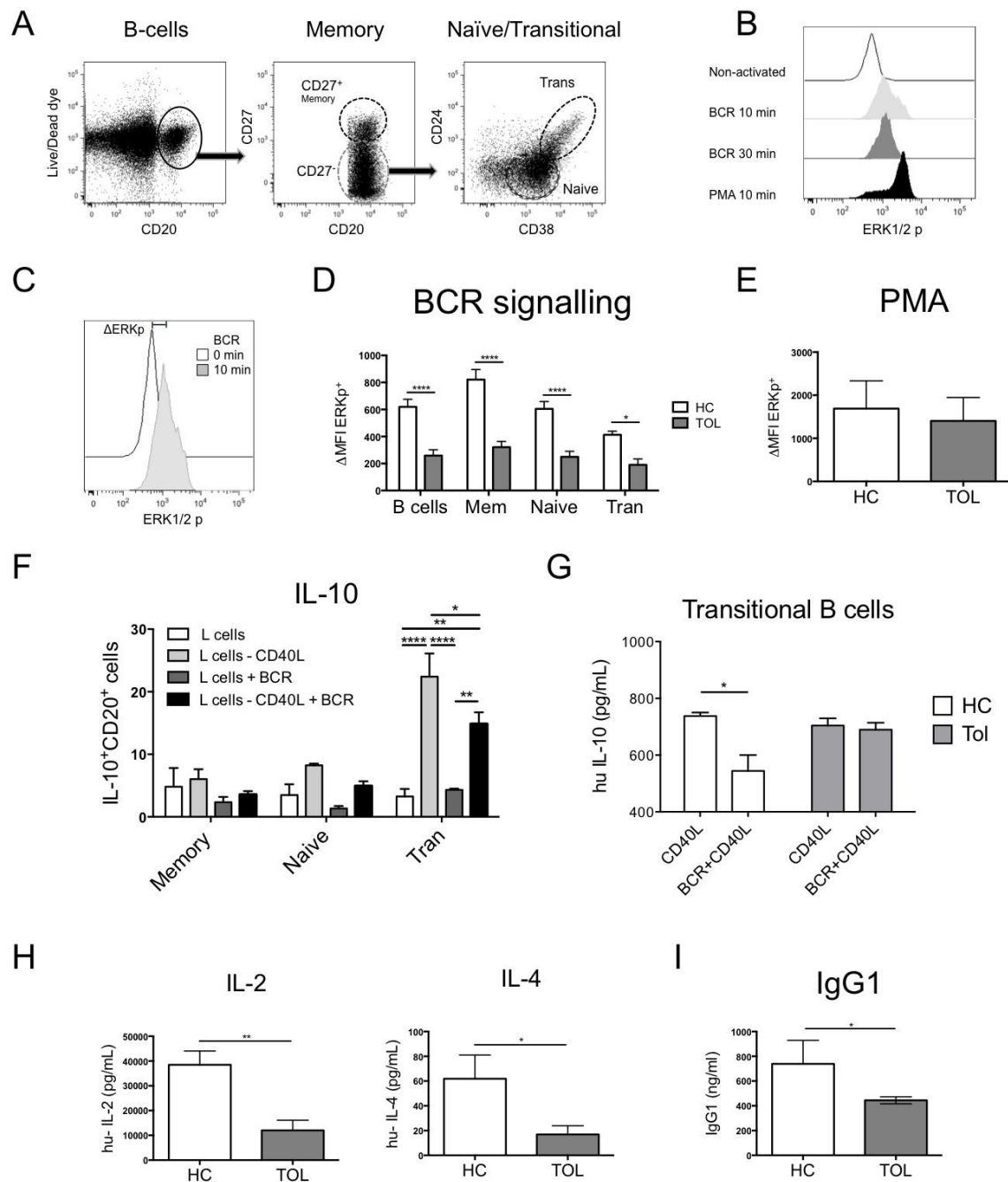


Fig. 2: B-cells from tolerant recipients display a reduced ERK signalling after BCR activation.

A) A phospho-flow B-cell panel was designed to identify p-ERK in B-cell subsets within PBMCs. B) A time course of p-ERK was measured in PBMCs after BCR-activation [anti-IgM (20 μ g/ml)/anti-IgG (20 μ g/ml)] during 0, 10 and 30min at 37°C, using PMA (0.1 μ M) for 10min as a positive control. C) Δ p-ERK was defined as the

difference between the p-ERK MFI from a non-activated and a 10min BCR-activated sample. D) The BCR signalling was measured in 1×10^6 PBMCs from healthy control (HC=9) and tolerant (Tol=9) patients after BCR-activation or after E) PMA ($0.1 \mu\text{M}$) for 10min. F) IL-10 production after BCR/CD40 activation was measured by adding 0.5×10^5 non-transfected or CD40L-transfected L-cells in 0.5×10^6 non-activated or BCR-activated B-cell subsets from healthy leukocytes retained in filtering cones after 3 days of culture by intracellular staining. G) Levels of IL-10 were measured in the supernatants of 10×10^4 sorted transitional B-cells from 3 HC and 3 Tol recipients activated with 1×10^3 non-transfected or CD40L-transfected L-cells, with or without BCR activation for 3 days. H) IL-2 and IL-4 production from isolated CD4⁺T-cells of HC=6 and Tol=7 were measured after 48h of activation with CD3/CD28 beads (1:2 ratio). Detection limit for IL-2 and IL-4 were 2.6 and 4.9pg/ml, respectively. I) Levels of IgG1 were measured in the sera from HC and Tol patients. Mann-Whitney test and two-way RM ANOVA test with a Sidak's multiple comparisons test were used, **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$ were considered significant.

Supplementary Methods

Intracellular Staining

Cells were activated with Phorbol 12-myristate 13-acetate (PMA) (50ng/mL, SIGMA), Ionomycin (1 μ g/mL, SIGMA), GolgiStop (1X, BD) and Brefeldin A (1X, BD) for 5hrs at 37°C 5% CO₂. Cells were stained with Live/Dead dye (Invitrogen), anti-CD20-AlexaFluor780 and anti-CD3-PerCP-Cy5.5 (all eBioscience) for 30min at 4°C. Samples were then fixed and permeabilized with FoxP₃ Fix and Perm Kit (BD) and stained with anti-IL10-PE (BD) and TNF- α (eBioscience) for 30min at 4°C. Cells were acquired on LSRFortessa (BD).

ELISA

ELISA (eBioscience: IL-10) (R&D: TNF- α) was performed according to manufacturer's instructions. Briefly, 96-well plates (Nunc™, Thermo Scientific) were incubated overnight with capture antibody at 4°C. The next day, plates were blocked for 1hr with 1%BSA, and standards and supernatant were added for 2hrs at room temperature. Detection antibody was added for 2hrs at room temperature and streptavidin-HRP was added for 20min at room temperature. Finally, plates were incubated with TMB (eBioscience) for 10min at room temperature and reaction was stopped using 0.5 M 2N H₂SO₄. Optical density at 405nm was measured using Sunrise ELISA Reader (TECAN, UK). The concentration of each cytokine was calculated from standard curves using Magellan data analysis Software. For IL-10: the sensitivity of the kit was 2 pg/mL and the standard curve range was from 2 to 300 pg/mL. For TNF- α : the sensitivity of the kit was 15.6 pg/mL and the standard curve range was from 15.6 to 1000 pg/mL.

B-cell subsets from peripheral blood cones

RosetteSep™ Human B cell enrichment cocktail (STEM CELL, UK) was used to obtain purified CD20⁺B-cells from leukocytes retained filtering cones from healthy volunteers blood donations (NHSBT Tooting blood bank, UK). B-cell subsets were sorted with ARIA II (BD). Memory B-cells were CD20⁺CD27⁺ (purity >95%), naïve B-cells CD20⁺CD27⁻CD24⁺CD38⁺ (purity >95%) and transitional B-cells CD20⁺CD27⁻CD24^{hi}CD38^{hi} (purity >95%). For IL-10 measurements in different B-cell subsets: 0.5x10⁶ B-cell subsets were cultured in complete media supplemented with IL-2 (100ng/ml, R&D) with or without 1.0x10⁴ plate bound CD40L-transfected and non-transfected mouse L fibroblast cells (X-ray irradiated for 30min 9,045 cGy), and CpG – a TLR9 agonist - (1μM; ODN 2006, InvivoGen) for 72hrs. After 3 days of activation, cells were stained with anti-CD20-AlexaFluor780 (eBioscience) and Live/Dead fixable yellow dead cell staining kit (Invitrogen) by surface staining and with IL-10-PE (BD) by intracellular staining for 30min at 4°C. For BCR signalling assays: 0.5x10⁶ non-activated or anti-IgM (20μg/ml)/anti-IgG (20μg/ml)-activated B-cell subsets were co-cultured with 0.5x10⁵ CD40L-transfected or non-transfected L-cells for 3 days. IL-10-PE (BD) was detected by intracellular staining. Cells were acquired on an LSRFortessa (BD). Data was analysed using FlowJo (Tree Star, Inc. Ashland, OR 97520. USA).

IL-10 production by transitional B-cells from patients and healthy controls

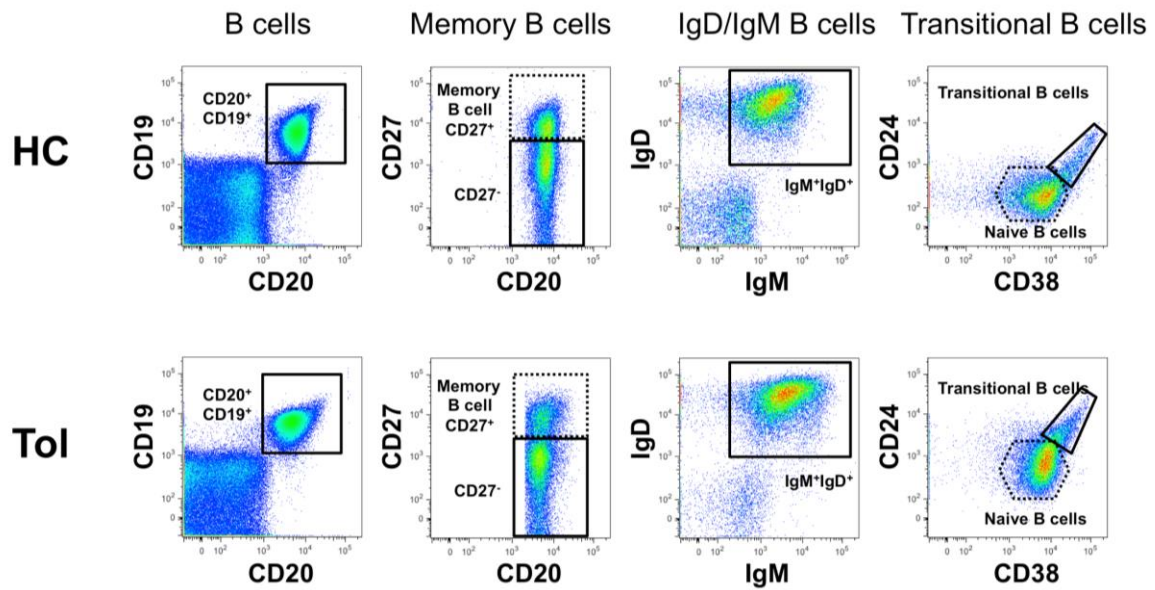
For IL-10 measurements in transitional B-cell subsets: 10x10⁴ sorted transitional B-cells from 3 healthy controls and 3 tolerant recipients were activated with 1x10³ non-transfected or CD40L-transfected L-cells, with or without BCR activation anti-IgM (20μg/ml)/anti-IgG (20μg/ml) for 3 days. IL-10 in the supernatants was measured using Cytometric Beads Array human Th1/Th2/Th17 kit (BD). For IL-10: the sensitivity of the

kit was 4.5 pg/mL and the standard curve range was from 4.5 to 5000 pg/mL. Beads were acquired on LSRFortessa (BD).

Flow Cytometry settings were used to monitor staining panels in healthy volunteers and patients samples

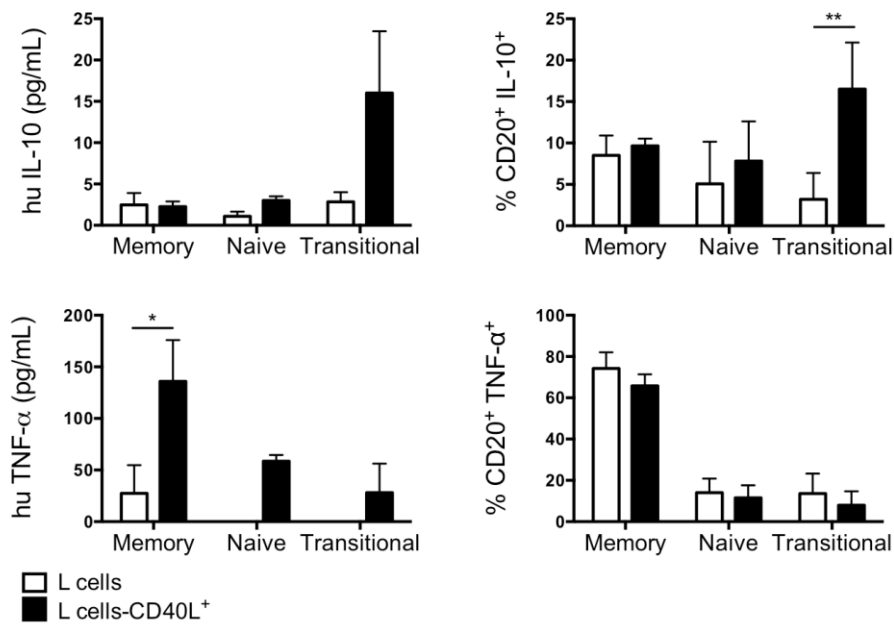
Flow cytometry panels in this study were monitored with CS&T (Cytometer Setup and Tracking, BD) beads, as a quality control for the Flow Cytometer; Application settings, as a quality control of the flow cytometry staining; and Inter and Intra-assay controls, as a quality control of the flow cytometry staining and the quality of the samples.

Supplementary Figures

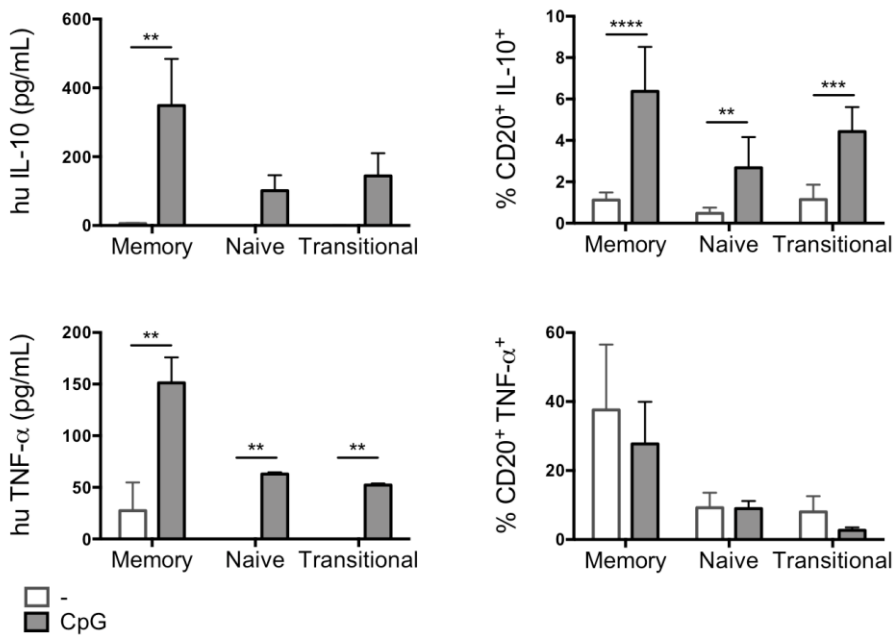


Supplementary Figure 1: B-cell subsets flow cytometry gating strategy. B-cells were identified as CD20⁺CD19⁺ B-cells. Within the B-cell population, memory B-cells were identified as CD27⁺ B-cells. IgM⁺IgD⁺ cells were gated from the CD27⁻ B-cell population and from the IgM⁺IgD⁺ B-cells, naïve B-cells were identified as CD24⁺CD38⁺ cells and transitional B-cells were identified as CD24^{hi}CD38^{hi} cells. Representative dot-plots of total, memory, IgD⁺IgM⁺, naïve and transitional B-cells were obtained from PBMCs from healthy controls (HC) and tolerant (Tol) patients by flow cytometry.

A

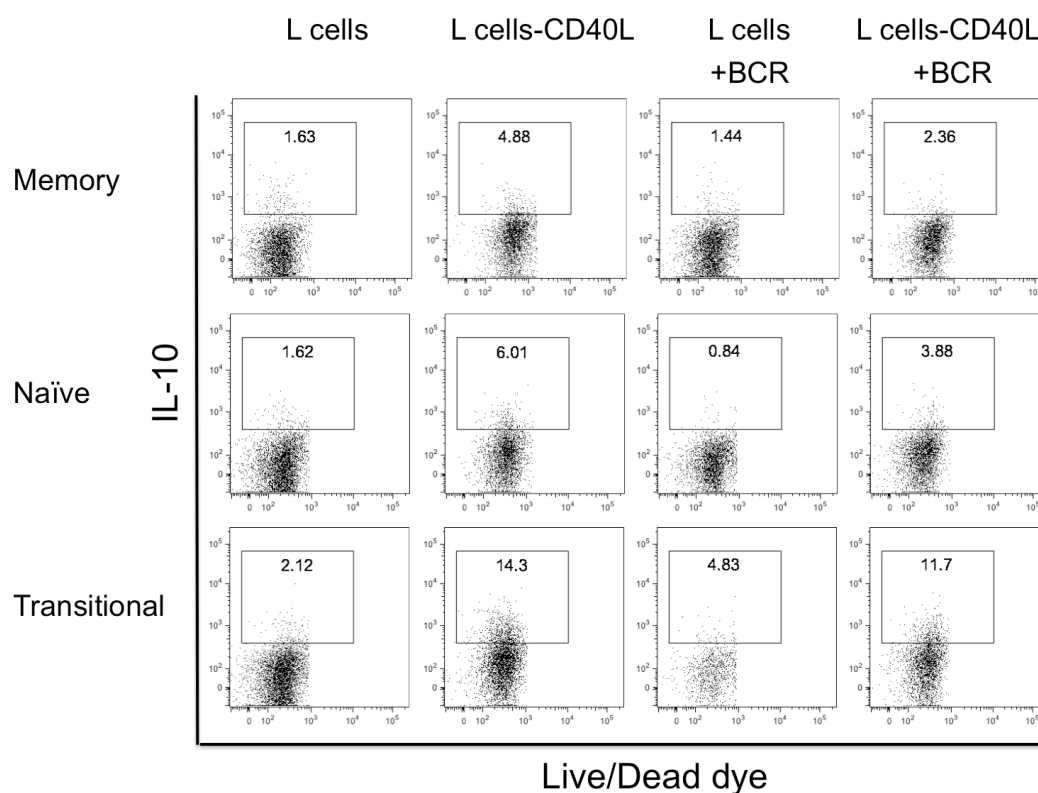


B



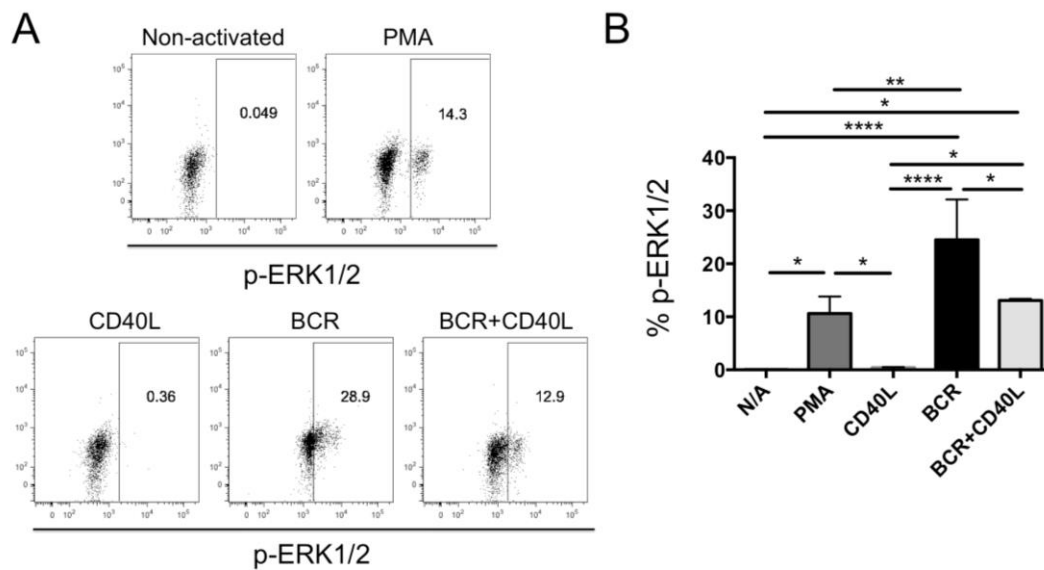
Supplementary Figure 2: IL-10 production by isolated B-cell subsets after CD40L and CpG activation. A) Sorted memory, naïve and transitional B-cells were obtained from healthy leukocytes retained in filtering cones. 0.5×10^6 sorted B-cell subsets were activated with 0.1×10^5 non-transfected L-cells or human-CD40L-transfected L-cells B) and with or without CpG ($1 \mu\text{M}$) for 72hrs. ELISA and intracellular

staining were used to measure IL-10 and TNF- α . Two-way RM ANOVA test with a Sidak's multiple comparisons test was used. For all statistical tests **** $P < 0.0001$, *** $P < 0.001$ and ** $P < 0.01$ were considered significant.



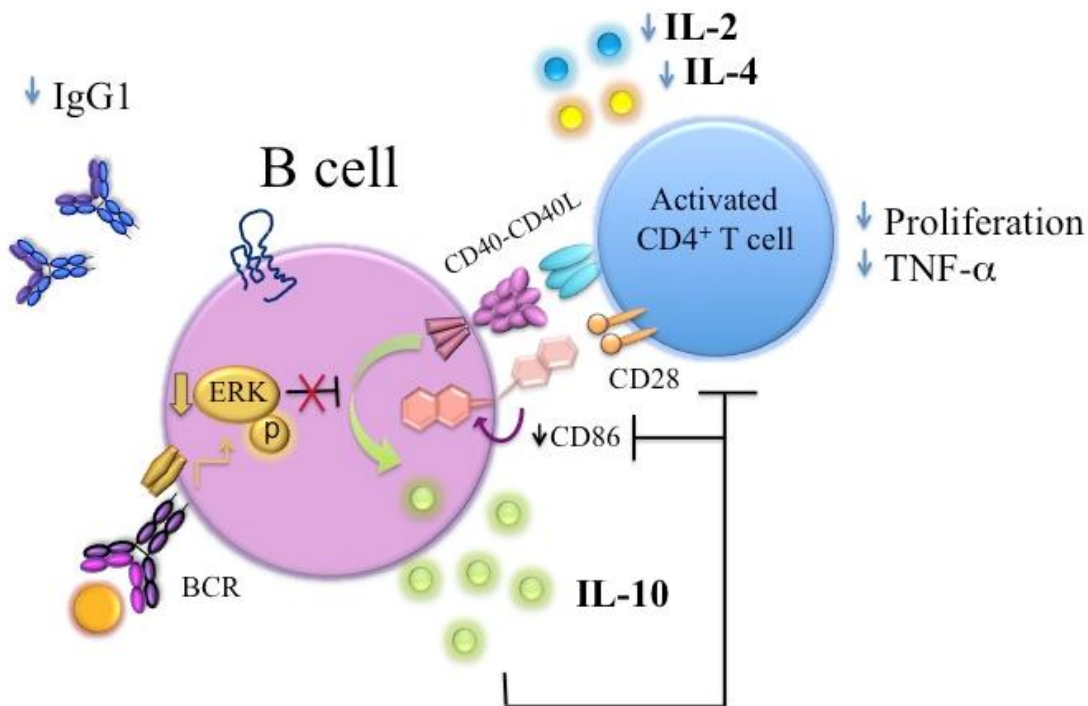
Supplementary Figure 3: IL-10 production by isolated B-cell subsets after CD40L, BCR and CD40/BCR activation.

IL-10 production after CD40, BCR and CD40/BCR activation was measured by adding 0.5×10^5 non-transfected or CD40L-transfected L-cells in 0.5×10^6 non-activated or BCR-activated sorted memory, naïve and transitional B-cells obtained from healthy leukocytes retained filtering cones. B-cell subsets were activated for 72hrs and IL-10 was detected with intracellular staining.



Supplementary Figure 4: ERK phosphorylation after BCR and CD40L activation.

A) Phosphorylation of ERK was assessed by phospho-flow using non-activated samples as negative controls and PMA-activated samples as positive controls. Phosphorylation ERK after CD40L, BCR, or BCR/CD40 activation was measured by adding 0.5×10^5 non-transfected or CD40L-transfected L-cells in 0.5×10^6 non-activated or BCR-activated B-cells from healthy volunteers after 3 days of culture by phospho-flow. B) ERK phosphorylation between different activating conditions was compared. Ordinary one-way ANOVA with a Tukey's multiple comparisons test was used, **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$ were considered significant.



Supplementary Figure 5: Schema of B-cell from tolerant recipients.

B-cells from tolerant patients exhibit higher percentages of IL-10-producing B-cells after CD40 activation with limited evidence of concomitant pro-inflammatory cytokines in parallel and reduced BCR signalling all when compared to B-cells from healthy controls. Reduction in the BCR signalling favoured IL-10 production in the transitional B-cell population of tolerant recipients. In addition, reduced IL-2 and IL-4 production by T-cells, may contribute to the low IgG1 levels observed in the serum. *Schema based on data from this manuscript and Nova-Lamperti et al., 2016¹⁴.*

Tolerant	Cause of end-stage renal disease	
Tol 1	Glomerular Disease	-Mesangial GN and Malignant Hypertension
Tol 2	Genetic Disorder	-Polycystic Kidney Disease
Tol 3	Other	-Unknown, possibly B-cell lymphoproliferative disorder
Tol 4	Other	-Traumatic loss of Kidney after Road accident
Tol 5	Other	-Unknown
Tol 6	Diabetes	-Diabetes type 2. Biopsy proven Nephropathy. Patient has Marfan Syndrome
Tol 7	Glomerular Disease	-Membranoproliferative GN Type 1
Tol 8	Pyelonephritis	-Pyelonephritis/Reflux Nephropathy
Tol 10	Glomerular Disease	-Membranous GN
Tol 11	Pyelonephritis	-Unknown, possibly chronic pyelonephritis
Tol 12	Genetic Disorder	-Congenital Renal Dysplasia
Tol 13	Glomerular Disease	-Membranoproliferative GN
Tol 14	Glomerular Disease	-Mesangial GN and Malignant Hypertension
Tol 15	Other	-Drug Induced ESRF post-leukemia
Tol 16	Glomerular Disease	-GN

Supplementary Table 1: Causes of Kidney failure or end-stage renal disease (ESRD) in tolerant kidney transplant recipients.

Tol: Tolerant recipient. GN: Glomerulonephritis. ESRF: End-stage Renal Failure.